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Biological Control of Aflatoxin Contamination in Corn Using a Nontoxigenic Strain of *Aspergillus flavus*

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ABSTRACT

A 2-year study was conducted to determine the efficacy of different applications of a nontoxigenic strain of *Aspergillus flavus* for reducing aflatoxin contamination in corn. Treatments consisted of the nontoxigenic strain in the form of (i) conidia-coated hulled barley applied to soil when corn was about 0.8 m tall, (ii) conidia-coated hulled barley applied in plant whorls prior to tasseling, (iii) multiple applications of a spray formulation of conidia during silking, and (iv) untreated control. Treatments were replicated eight times in individual plots consisting of four rows of 18 m each. In year 1, no significant differences were associated with treatments for aflatoxin, total *A. flavus* colonization, or incidence of nontoxigenic isolates of *A. flavus* in corn, which were all relatively high, ranging from 83.8 to 93.1%. In year 2, the whorl application produced a significantly lower mean aflatoxin concentration of 49.5 ppb compared with all other treatments, while both the soil (108.3 ppb) and spray applications (173.7 ppb) were significantly reduced compared with the control (191.6 ppb). The whorl application was the only treatment that had a significantly higher incidence (86.5%) of nontoxigenic isolates of *A. flavus* than the control had, which was still relatively high at 69.1%. Data indicated that applications of the nontoxigenic strain influenced untreated corn, thus reducing the apparent effect of the biocontrol treatments. Larger-scale studies with greater separation between treated and untreated fields are warranted.

Aspergillus flavus Link and *Aspergillus parasiticus* Speare are fungi that are capable of invading various food and feed crops and contaminating them with hepatotoxic and carcinogenic aflatoxins *(7).* In addition to the safety hazard posed by aflatoxin contamination, these fungi place a significant economic burden on food and feed industries to ensure that contaminated products do not enter the food and feed supply *(13).* Crops that are particularly affected include corn, peanut, cottonseed, and various tree nuts. In recent years, biological control technology based on competitive exclusion has been developed to control aflatoxin contamination *(3).* Biocontrol is achieved by establishing a non-aflatoxigenic strain of *A. flavus* or *A. parasiticus* in the soil of a developing crop, which then displaces or excludes toxigenic strains during crop infection and colonization. Two such products have been registered by the U.S. Environmental Protection Agency as biopesticides to control aflatoxin. One is afla-guard for aflatoxin control in peanuts *(16),* and the other is *Aspergillus flavus* AF36 for control of aflatoxin in cottonseed *(15).* Afla-guard is composed of hulled barley coated with conidia of a nontoxigenic strain of *A. flavus* (NRRL 21882) that does not produce aflatoxins, cyclopiazonic acid, or known aflatoxin biosynthetic precursors *(4).* Large-scale tests of peanuts showed that applications of afla-guard produced reductions in aflatoxin, averaging 85.2% in farmers' stock peanuts and as high as 97.5% in shelled, edible grade peanuts *(6).* The objective of this study was to evaluate the efficacy of *A. flavus* NRRL 21882 with three different inoculation techniques to control aflatoxin contamination in corn.

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MATERIALS AND METHODS

Field study design. The 2-year study used a randomized complete block design with two corn plantings, four biocontrol treatments, and eight replications. Dekalb DKC67-60, a Roundup Ready (Monsanto, Creve Coeur, MO) hybrid lacking enhanced insect resistance, was planted on 4 and 24 March 2005, and 7 and 28 March 2006, in 64 individual plots consisting of four, 18-m rows spaced 91 cm apart in an otherwise-uncultivated field located about 11 km west of Dawson, GA. Distance between plots was 3.7 m side to side and 12.2 m end to end. Treatments included (i) untreated control, (ii) afla-guard applied to soil at 22.4 kg/ha, (iii) afla-guard placed in plant whorls at the same rate, and (iv) an aqueous conidial suspension of the nontoxigenic *A. flavus* applied four times during silking. Soil applications were made with a tractor-pulled granular applicator when plants were approximately 0.8 m tall. Whorl applications were made by hand shortly before tasseling, at the time of maximum whorl opening. The conidial suspension was prepared by first suspending 2 to 3 g of dry conidia of the nontoxigenic *A. flavus* in 100 ml of the nonionic surfactant DyneAmic (Helena Chemical Co., Collierville, TN) and diluting that in water to give a final concentration of $1.0 \pm 0.4 \times 10^6$ conidia per milliliter. The suspension was applied four times on alternate days as a spray from above the plants, beginning at the time of first silk at a rate of 123 liters/ha. In 2005, both plantings were harvested on 17 August (146 and 166 days after planting, respectively) with a commercial combine. All plots within a treatment were harvested consecutively, and corn from the second half of each plot was collected for processing. In 2006, all ears were harvested by hand on 16 August (first planting, 162 days after

TABLE 1. *Mean aflatoxin concentrations in corn from two planting dates and four biocontrol treatments in 2005*

Treatment	First planting (ppb)	Second planting (ppb)
Control	35.6 A[a]	96.6 A
Soil[b]	25.8 A	48.6 A
Whorl[c]	31.5 A	78.7 A
Spray[d]	30.6 A	130.3 A

[a] Means that are followed by the same letter in a column are not significantly ($P > 0.05$) different.
[b] Afla-guard applied to soil at 22.4 kg/ha.
[c] Afla-guard applied in plant whorls at 22.4 kg/ha.
[d] Conidia of *Aspergillus flavus* NRRL 21882 applied as a broadcast spray application from above plants four times during silking.

TABLE 2. *Mean aflatoxin concentrations in corn from two planting dates and four biocontrol treatments in 2006*

Treatment	First planting (ppb)	Second planting (ppb)	Overall (ppb)
Control	185.9 A[a]	197.3 A	191.6 A
Soil[b]	83.0 B	133.5 A	108.3 B
Whorl[c]	44.4 B	54.6 B	49.5 C
Spray[d]	62.4 B	285.1 A	173.7 B

[a] Means that are followed by the same letter in a column are not significantly ($P > 0.05$) different.
[b] Afla-guard applied to soil at 22.4 kg/ha.
[c] Afla-guard applied in plant whorls at 22.4 kg/ha.
[d] Conidia of *Aspergillus flavus* NRRL 21882 applied as a broadcast spray application from above plants four times during silking.

planting) and 30 August (second planting, 155 days after planting), because severe drought greatly reduced yield.

Corn processing. In 2005, samples from each plot, averaging 22 kg, were ground in a Romer Series II subsampling mill (Romer Labs, Inc., Union, MO) that was set to provide a subsample of approximately 3.3 kg (15%). Because of reduced yield in 2006, samples averaged 6.1 kg, with subsamples averaging 2.3 kg. Each subsample was ground with an equal weight of water in a Stephan VCM12 vertical cutter mixer (Sympak Inc., Mundelein, IL) for 7 min to produce a homogeneous slurry before taking subsamples for fungal and aflatoxin analyses.

Fungal analyses. Two hundred grams of slurry was added to 200 ml of water and blended at low speed in an autoclaved, stainless steel blender for 1 min *(1).* Serial dilutions were plated on modified dichloran–rose bengal medium *(9, 12)* and incubated for 3 days at 37°C. Colonies of *A. flavus* and *A. parasiticus* were identified and counted directly on the plates to determine CFU per gram. This method was used to quantify the colonization of corn by *A. flavus* rather than plating individual kernels to determine infection frequency, because previous studies with peanuts showed it to have a much higher correlation with aflatoxin contamination than did infection frequency *(1).* To determine the incidence of nontoxigenic isolates of *A. flavus* in corn, 10 random isolates per sample were cultured and analyzed for aflatoxins and cyclopiazonic acid, as previously described *(10, 11).* Briefly, *A. flavus* isolates were cultured for 1 week on 1 ml of yeast extract–sucrose liquid medium in 4-ml vials at 30°C. Cultures were extracted with 1 ml of chloroform and subjected to thin-layer chromatography and high-performance liquid chromatography analyses.

Aflatoxin quantitation. A separate 200-g aliquot of the corn slurry was added to 400 ml of methanol and blended for 1 min *(1).* The filtered extract was cleaned on a minicolumn packed with basic aluminum oxide and subjected to high-performance liquid chromatography analysis, as described by Sobolev and Dorner *(14).*

Additional field samples. In 2006, six additional corn samples, averaging 13 kg, were collected from a field located 3.7 km from the study site. They were processed and analyzed for aflatoxin and fungal colonization, as described for other samples.

Statistics. Data were log transformed where necessary to normalize distributions and subjected to two-way analysis of variance. Means were separated with the Student-Newman-Keuls method at $P < 0.05$, using SigmaStat for Windows, version 3.5 (Systat Software, Inc., San Jose, CA).

RESULTS

Aflatoxin contamination. In 2005, there was a significant ($P = 0.035$) planting date effect on aflatoxin contamination, but there was no effect from the different treatments and no interaction between planting date and treatment. Corn from planting date 1 averaged 30.9 ppb of aflatoxin, while corn from date 2 averaged 88.5 ppb. Aflatoxin concentrations for each treatment from each planting are given in Table 1, with no significant differences observed.

A severe drought in 2006 had the dual effect of significantly ($P < 0.001$) reducing yield (6.1 kg per plot in 2006 compared with 22 kg per plot in 2005) as well as significantly ($P < 0.001$) increasing overall mean aflatoxin concentrations (60 ppb in 2005 compared with 131 ppb in 2006). As in 2005, there were significant effects on aflatoxin contamination by both planting date ($P < 0.001$) and treatment ($P < 0.001$) in 2006, with no significant interaction between date and treatment. Mean aflatoxin concentrations for planting dates 1 and 2 were 93.9 and 167.6 ppb, respectively. Aflatoxin data for each treatment are given in Table 2. In the first planting, all treatments produced significant reductions in aflatoxin compared with the control. In the second planting, only corn from the whorl application treatment contained significantly less aflatoxin. When data for both plantings were analyzed together, each treatment produced significant reductions compared with the control, with the whorl application treatment producing the greatest reduction.

Fungal colonization. In 2005, there were no significant differences among treatments for total *A. flavus* colonization of corn or for the incidence of nontoxigenic isolates of *A. flavus* in corn (Table 3). Total *A. flavus* in corn was about an order of magnitude higher in 2006 than in 2005, but was again not significantly affected by biocontrol treatments (Table 3). However, in 2006, the whorl application of afla-guard yielded a significantly higher percentage of nontoxigenic isolates (86.5%) than did the control

TABLE 3. *Total* Aspergillus flavus *colonization of corn and incidence of nontoxigenic isolates in 2005 and 2006*

Treatment	2005		2006	
	CFU/g	% nontoxigenic	CFU/g	% nontoxigenic
Control	2.1×10^5 A[a]	84.3 A	4.2×10^6 A	69.1 A
Soil[b]	2.4×10^5 A	83.8 A	3.8×10^6 A	79.4 AB
Whorl[c]	2.9×10^5 A	93.1 A	2.5×10^6 A	86.5 B
Spray[d]	4.5×10^5 A	91.9 A	5.2×10^6 A	72.5 A

[a] Means that are followed by the same letter in a column are not significantly ($P > 0.05$) different.
[b] Afla-guard applied to soil at 22.4 kg/ha.
[c] Afla-guard applied in plant whorls at 22.4 kg/ha.
[d] Conidia of *Aspergillus flavus* NRRL 21882 applied as a broadcast spray application from above plants four times during silking.

and spray application treatments. There was no significant difference in incidence of nontoxigenic isolates between soil and whorl applications of afla-guard.

Additional field samples. Additional samples were collected in 2006 from a field in the same area (3.7 km away) and exposed to essentially the same weather conditions as was the study field. The purpose was to gain information on aflatoxin contamination and *A. flavus* colonization from corn that was not under any influence from the application of the nontoxigenic strain. The mean aflatoxin concentration in these samples was 276 ± 37 ppb, which was significantly ($P = 0.043$) higher than was the mean of 192 ppb for the control corn at the study site. Mean *A. flavus* colonization for the additional samples was 8.6×10^5 CFU/g, and the incidence of nontoxigenic isolates averaged 20% ± 10%.

DISCUSSION

Previous studies have demonstrated the efficacy of afla-guard for biological control of aflatoxin contamination of peanuts *(6).* In the current study, afla-guard showed potential for controlling aflatoxin contamination in corn as well. This conclusion is not obvious from a cursory examination of the aflatoxin data, particularly for 2005, during which no significant differences in aflatoxin contamination were observed. Significant reductions were found in 2006, with a maximum reduction of 76% for the whorl application in the first planting. A closer examination of all data indicates that the various treatments with the nontoxigenic *A. flavus* likely had a mitigating effect on aflatoxin contamination in the untreated corn as well as corn that was directly treated. In 2005, there were no differences in the incidence of nontoxigenic isolates of *A. flavus* in any of the treatments, with control corn having a very high incidence of 84.3%. There was also no difference in total *A. flavus* colonization of corn among treatments. In 2006, the incidence of nontoxigenic isolates in controls averaged 69.1%, and the only treatment that was significantly higher was the whorl application treatment. The logical reason for the unusually high incidence of nontoxigenic isolates in untreated corn is that the close proximity of treated and nontreated plots facilitated spread of the inoculum into the controls. In the randomized complete block design, plots were separated by only 3.7 m side to side and 12.2 m end to end. In a previous, similarly designed study testing different biocontrol formulations in peanuts, significant differences were found between controls and treatments for both aflatoxin contamination and the incidence of nontoxigenic isolates of *A. flavus (2).* However, peanuts are produced in the soil, where it is perhaps easier to effect and maintain a change in the composition of the *A. flavus* population than it is in air, where corn ears are forming and maturing. Conidia of *A. flavus* produced on the surface of afla-guard granules become airborne and may be blown by wind and carried by insects away from plots to which they were applied *(8).* The high incidence of the nontoxigenic strain in corn from control plots indicates there was significant infiltration of conidia from surrounding treated plots into untreated controls. This view is further supported by data collected from corn grown in a field 3.7 km away from the study site. This corn was grown during the same period and subjected to very similar environmental conditions as corn at the test site. It contained significantly more aflatoxin than did control corn at the test site and had a much lower incidence (20%) of nontoxigenic isolates of *A. flavus.* Therefore, even though significant reductions in aflatoxin were achieved with all biocontrol treatments in 2006, it is probable that reductions would have been greater without the influence of the nontoxigenic strain in the untreated corn.

In an earlier 4-year study testing this concept of biological control in corn, aflatoxin reductions of up to 87% were achieved with a combination of nontoxigenic strains of *A. flavus* and *A. parasiticus* applied as colonized rice to soil at 225 kg/ha *(5).* That study demonstrated potential for biocontrol of aflatoxin in corn, but it did so with application rates that were impractically high for commercial use. The current study sought to test for biocontrol in corn by using commercially available afla-guard applied at an economically practical rate (22.4 kg/ha). It also tested the efficacy of three different modes of application. Results indicate that significant reductions in aflatoxin can be achieved, and that whorl application may provide the greatest degree of control. Based on these results, it is recommended that in commercial corn production, afla-guard be aerially applied prior to tasseling, when corn plant whorls are maximally open. In that scenario, many granules would fall to the soil surface, but it is also likely that many would be caught in plant whorls. These results warrant large-scale studies in which entire fields are treated and compared with nearby untreated fields, so that a more accurate assessment of efficacy can be determined.

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